SOME EFFECTS OF VARYING DIETARY VITAMIN C LEVELS ON THE REDUCING CAPACITY OF THE ADRENAL GLANDS AND THE FUNCTION OF THESE BODIES UNDER STRESS

by

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INTRODUCTION

Ascorbic acid has long been associated with the adrenal cortex. This vitamin was first obtained (31) and identified (12, 13, 33) from the adrenal cortex about 20 years ago. Since then, numerous studies have probed the role of the vitamin at this site. It has been suggested (10, 23, 24, 27) that ascorbic acid may act as a coenzyme in the biological oxidation which produces the oxy-type of the adrenal steroids.

Several years ago, Heard, Sobel and Venning (8, 9) applied the reduction of phosphomolybdic acid forming molybdenum blue to the quantitative estimation of small quantities (100 gamma) of reducing steroids of the adrenal cortical class. The degree of reduction as recorded by the spectrophotometer due to these steroids is an inverse measure of the oxidation state of these steroids.

The effect of varying vitamin C in the diet of the albino rat upon the reducing capacity of the adrenal steroids was tested in our experiment by the above procedure. Should the vitamin act as a coenzyme in the production of the oxy-type steroids, a trend toward decreasing reduction should be noted at higher dietary vitamin C levels.

Further, this gland is intimately involved with the general-adaptation-syndrome (28), a series of morphological and biochemical changes governed by the adrenal cortex and stimulated by non-specific stresses (29). Numerous studies have been made of the effects of stress upon the secretion of various adrenal substances. These affirm a marked ascorbic acid loss by the adrenal cortex following ACTH administration (10). Further there is a 700 per cent increase in the adrenocortical secretion of the 11-oxysteroids (32). These reactions would be expected if the coenzymatic role postulated above for the vitamin were valid.

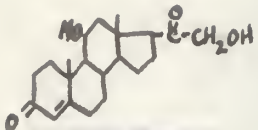
If such a role does exist, increased vitamin C should result in increased cortical hormone. Giroud and co-workers noted that cortical hormone production was enhanced by vitamin C administration (3, 4, 5). Further, increased ascorbic acid intake, in providing more hormone, should enable an animal to withstand stress better. Large doses of ascorbic acid intake in the rat and guinea pig completely prevent the adrenal hypertrophy normally resulting from chronic exposure to cold (2). In the second phase of this experiment we tested the response of rats on varied dietary vitamin C levels to etherization stress (14).

HISTORICAL BACKGROUND

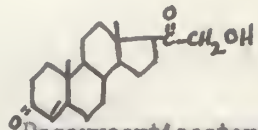
About 28 individual crystalline compounds have been isolated from extracts of the adrenal cortex. Exhaustive studies have characterized these as steroids (17, 18, 19, 25, 26, 34, 35). Five have been characterized as being profoundly physiologically active, but their activities vary widely according to the response being tested. In addition, an amorphous residue is left from these extracts which is yet uncharacterized and which shows considerably more activity than the crystalline substances. The formulae of these five identified active principles are shown on the following page.

Giroud and Santa (3, 4, 5) repeatedly observed that ascorbic acid administration favored the production of adrenal hormone in the guinea pig as determined by the carp-scale melanophore - contraction reaction.

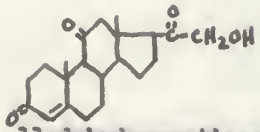
These workers also claim (6) ascorbic acid is necessary for the normal secretion of cortical hormone. This secretion increases with increased ascorbic acid content. The correct functioning of the cortical cells demands a certain optimum level of vitaminization.



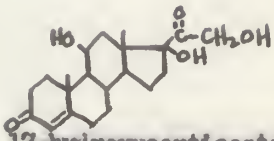
Corticosterone



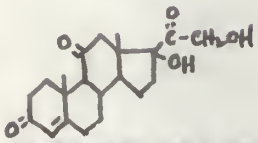
Desoxycorticosterone



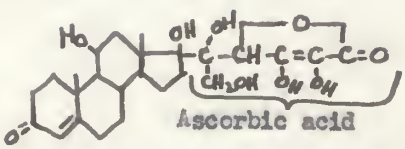
11-dehydrocorticosterone



17-hydroxycorticosterone



11-dehydro,17-hydroxycorticosterone
(Cortisone, Reichstein's Compound F,
Kendall's Compound E)



Intermediate proposed by Lowenstein
and Zwemer (16)

A number of considerations suggest that the true nature of the cortical hormone may be a glycoside with the steroid moiety as the aglycone. It may be linked to either a carbohydrate or ascorbic acid. Evidence for such a combination is threefold:

1. The isolation of such a substance from the gland. Lowenstein and Zwemer (16) have isolated such a compound from aqueous extracts of the adrenals. The formula of this compound is shown on the preceding page.

2. The physiological activity of similar substances. The naturally occurring cardiac glycoside, strophanthin, was suggested by Fieser as bearing a close resemblance to the structure of the above postulated intermediate (36). The following similarities were noted between cortin and this and other cardiac glycosides:

ACTIVITY	CORTIN	CARDIAC GLYCOSIDES
Lowers plasma potassium	yes	yes
Lowers plasma spec. grav. (proteins)	yes	yes
Raises blood sugar	yes	yes
Favors polymorph increase	yes	yes
Protection against lethal potassium	yes	yes
Protection against insulin death	yes	yes
Protection against diphtheria toxin	yes	yes

3. Adrenal steroids display a more pronounced effect on the carbohydrate metabolism of the rat (15) than in man (7). This difference may lie in the ability of the rat to synthesize its own ascorbic acid. Since this early work several steroid glycosides have been isolated, synthesized, and are believed to occur naturally in this form. They are the tetraacetyl-β-D-glucoside of desoxycorticosterone (11) and desoxycorticosterone-6-(B-lactosido)-D-glucoside and its derivatives (22).

EXPERIMENTAL PROCEDURE
PHASE I

Animals Used. Fifty young male albino rats, weighing 140-160 grams were purchased from Sprague Dawley Inc., Madison, 5, Wisconsin. They were maintained in individual rat cages with wire bottoms. They were watered daily and fed every two days, 29 grams at each feeding, enough to insure normal growth with no excess left in the dishes after 48 hours.

Rations. The basal ration was ground lab chow pellets (PURINA). The various levels were supplemented with ascorbic acid amounting to 0, 15, 30, 45, and 60 mg./day/rat. These ration mixtures were prepared at ten day intervals.

Sacrifice of Animals. After 20 days on such rations, the animals were sacrificed by injection of 1 cc. of a pentobarbital sodium solution in alcohol (NEMBUTAL). This manner of sacrifice was adopted as one which could be uniformly administered to all animals, and which is not an adrenal stress (14) causing alarm reaction secretions (19, 25).

Preparation of Gland Tissue. The adrenals were removed within 30 minutes following the injection. After being weighed, they were placed in 1 cc. ether-chloroform (4:1) solution in individual 3" glass test tube, stoppered, and stored in a freezer until all the adrenals had been removed. They were subsequently ground in the ether-chloroform mixture in the same tubes by an electrically powered revolving glass rod. The samples were replaced in the freezer and allowed to stand for one week by which time the undissolved material had settled out, mostly clinging to the sides of the tube. Under these conditions, desoxycorticosterone and other steroids remain stable for many months (8). The ether-chloroform solution was then decanted for fractionation.

Fractionation. The ether-chloroform solution was decanted into a 10 cc. graduated cylinder and diluted to 3.0 cc. with ether-chloroform solution. This was then shaken with 2.5 cc. of cold 0.1 N Sodium hydroxide solution (aqueous). Two layers formed. One cc. of the clear lipid fraction (top) was pipetted into a clean 3" glass test tube where it was permitted to evaporate. The residue was dissolved in 3 cc. glacial acetic acid.

The water soluble fraction required 10-15 minutes to clear. One cc. of the clear solution was removed by pipette and placed in a test tube. Just prior to analysis 2 cc. of glacial acetic acid were added.

Analysis of Steroid Reducing Capacity - Theory. The reduction of phosphomolybdic acid in an acetic acid medium by adrenal steroids is not appreciably influenced by atmospheric oxygen. The development of the molybdenum blue color proceeds rapidly (within a half hour) at 100° C. After this, the intensity gradient falls off sufficiently to permit obtaining reproducible results at any arbitrarily chosen time thereafter. Heard and Sobel (8) chose 1 hour as the heating time and the same period was adopted in our studies. Under these conditions, optical density readings at the same concentrations agree within ±2%. Further, Beer's Law has been verified for the analysis by this method of a large number of steroids, including cortisone and desoxycorticosterone. German workers have used the same analysis (30).

The strongest reducing capacity of the ketosteroids was exhibited by the primary α-ketol group which is attached to the 17 position of all these compounds.

A tertiary hydroxyl group at C_{17} as in cortisone is non-reducing. Actually, its presence diminshes somewhat the reducing power of the α-ketol group (8). Acetylation of the primary alcohol also slows reduction.

Oxidation, forming a ketonic oxygen at C_{11} or C_{12}, adversely influences the reducing capacity.

In view of these effects, if vitamin C contributes to the oxidation of positions 11 or 17, reduced reduction is expected.

No work has been reported on the effect of the glycoside linkage on the reduction by steroids.

Preparation of Reagent. 100 g. of sodium molybdate were dissolved in 200 cc. distilled water, and 110 cc. of 85% H_3PO_4, 60 cc. distilled water, 20 cc. concentrated H_2SO_4 and 40 cc. glacial acetic acid were added with stirring.

Method of Color Development and Determination. It was found advisable to perform the following procedure on all the water fraction samples and blank as a group and proceed similarly with the lipid fractions. This procedure avoided the preparation of an excessive number of blanks and frequent alterations of zero readings on the colorimeter.

Three cc. of the sample fraction to be analyzed were introduced into the bottom of a 6" test tube. Two cc. of the reagent were then added and, after admixture by shaking, the tube was placed in a boiling water bath for exactly 60 minutes. On removal it was immediately cooled (30 seconds in a stream of cold tap water), and the contents were quantitatively transferred with 8 cc. of the reagent to one of a paid of matched colorimeter tubes. The air bubbles which form when the reagent is added required about 90 seconds to rise to the surface because of the viscosity of the solution. The optical density was then read within the next four minutes at 655 millimicrons.

A blank determination with 3 cc. of the glacial acetic acid (or 2 cc. glacial acetic acid plus 1 cc. of 0.1 N N_aOH solution for the aqueous blank) and 2 cc. of the reagent was run in the same manner, serving as controls

for the adjustment of the colorimeter to zero optical density. The blank solution absorbed very slightly in the range measured, but the optical density remained constant regardless of the period of heating.

EXPERIMENTAL PROCEDURE
PHASE II

Animals Used. 24 albino rats of both sexes from our own laboratory stock weighing 150-200 g. were used. Six groups of 4 different dietary ascorbic acid levels each were maintained. The four animals in each group were, as far as possible, litter mates of the same sex. They were maintained in individual rat cages with wire bottoms. They were watered daily and fed every two days, 29 grams of feed at each feeding - an amount sufficient to insure normal growth with no excess feed left in the dishes at the end of the 48 hour period.

Rations. The basal ration was ground lab chow pellets (PURINA). The various levels were supplemented with ascorbic acid amounting to 0, 5, 10, and 15 mg./day/rat during the second portion of this phase. These ration mixtures were prepared at 10 day intervals.

Treatment of Animals. No supplementary ascorbic acid was fed to any of the rats for 25 days. After this period they were etherized three times and their recovery times noted. Afterwards the animals were placed on different levels of the nutrient for 25 days and were etherized three times as before. About 90 minutes elapsed between subsequent etherizings on the same animal.

The animals were etherized in 6 groups of 4 animals each. The rats, one from each dietary level, were placed in an etherizing dessicator together, in this way subjected to equal concentrations of ether. Fifty

cc. of ether were placed in the bottom of the dessicator and the lid closed. The animals were left in the ether atmosphere for 2 minutes, removed and placed in a liberal supply of fresh air. The "recovery time" was measured as the time elapsed between removal of the animal from the dessicator and the first bodily movements which persisted for 5 seconds. (Reflex twitches generally last less than 5 seconds.) Choosing an indication of consciousness is quite arbitrary.

STATISTICAL METHODS EMPLOYED
PHASE II

For various reasons, such as the difference in age of rats between the first and second treatments of ether, ether atmosphere differences in the dessicator, etc., there resulted a longer recovery period the second time the rats were etherized. One rat in each of the six groups received no supplementary vitamin C at any time. Since it was subject to the same conditions as the other three of its group, it was assumed that the difference in its recovery time would have been the same as the other three members if they had received no vitamin C. Therefore it was used as a control.

For example, if under one set of etherizing conditions, a control rat took 110 seconds and under the second took 94 seconds, 16 seconds would be added to the second readings of all rats in that same group. This 16 seconds would be our "correction factor" for that entire group.

EXPERIMENTAL RESULTS - PHASE I

For the aqueous fraction, as the dietary vitamin C increased, the optical density of the molybdenum blue solution formed (degree of reduction) per 100 mg. adrenal tissue decreased (Fig. 1). There was no apparent difference resulting from ascorbic acid dietary level differences in the lipid fraction (Fig. 2). (Also see tables 1 and 2).

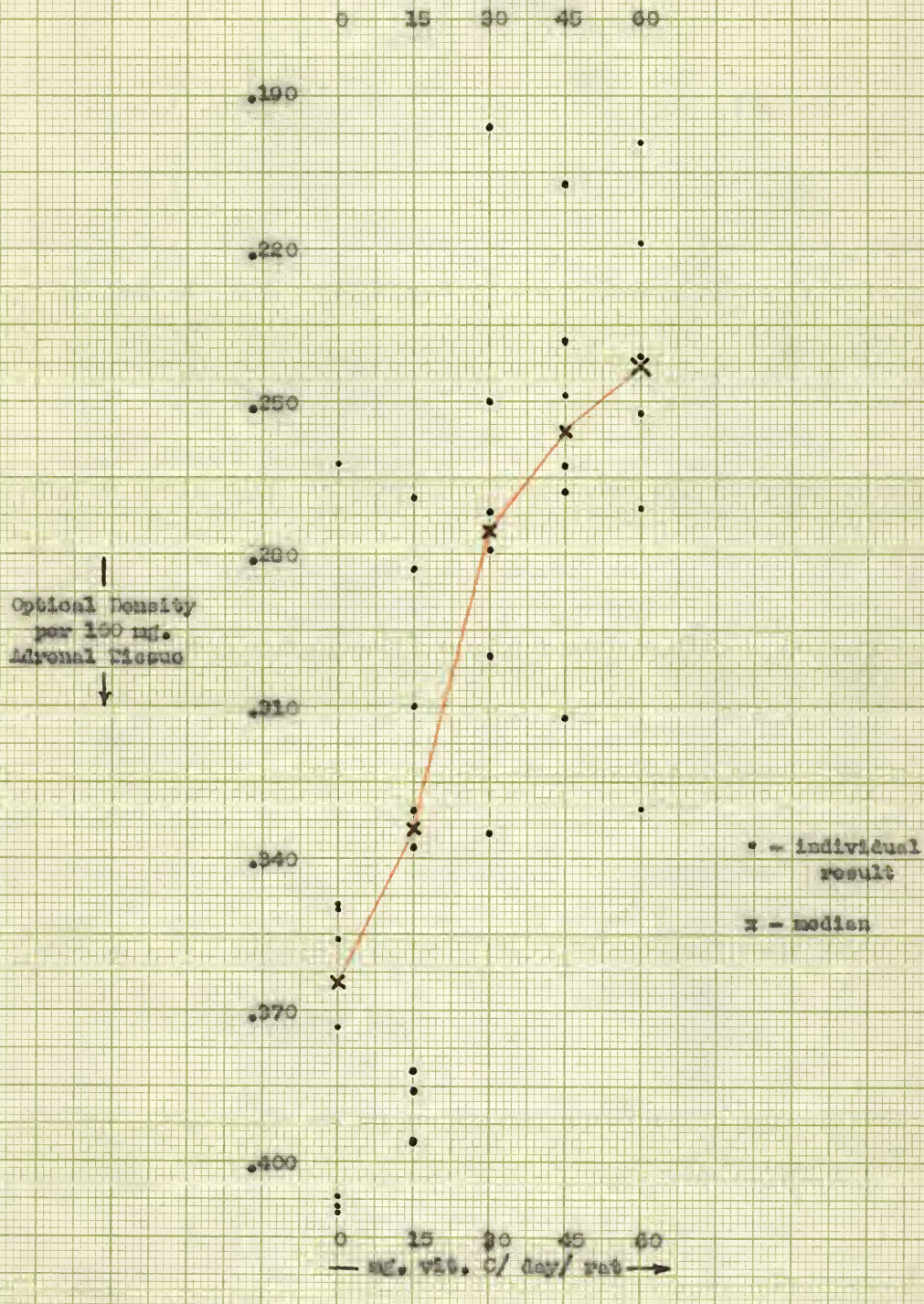

0 15 30 45 60
.190
.220
.250
.280
.310
.340
.370
.400
Optical Density per 100 mg. Adrenal Tissue
• = individual result
x = median
0 15 30 45 60
— mg. vit. C/ day/ rat →

Figure 1.

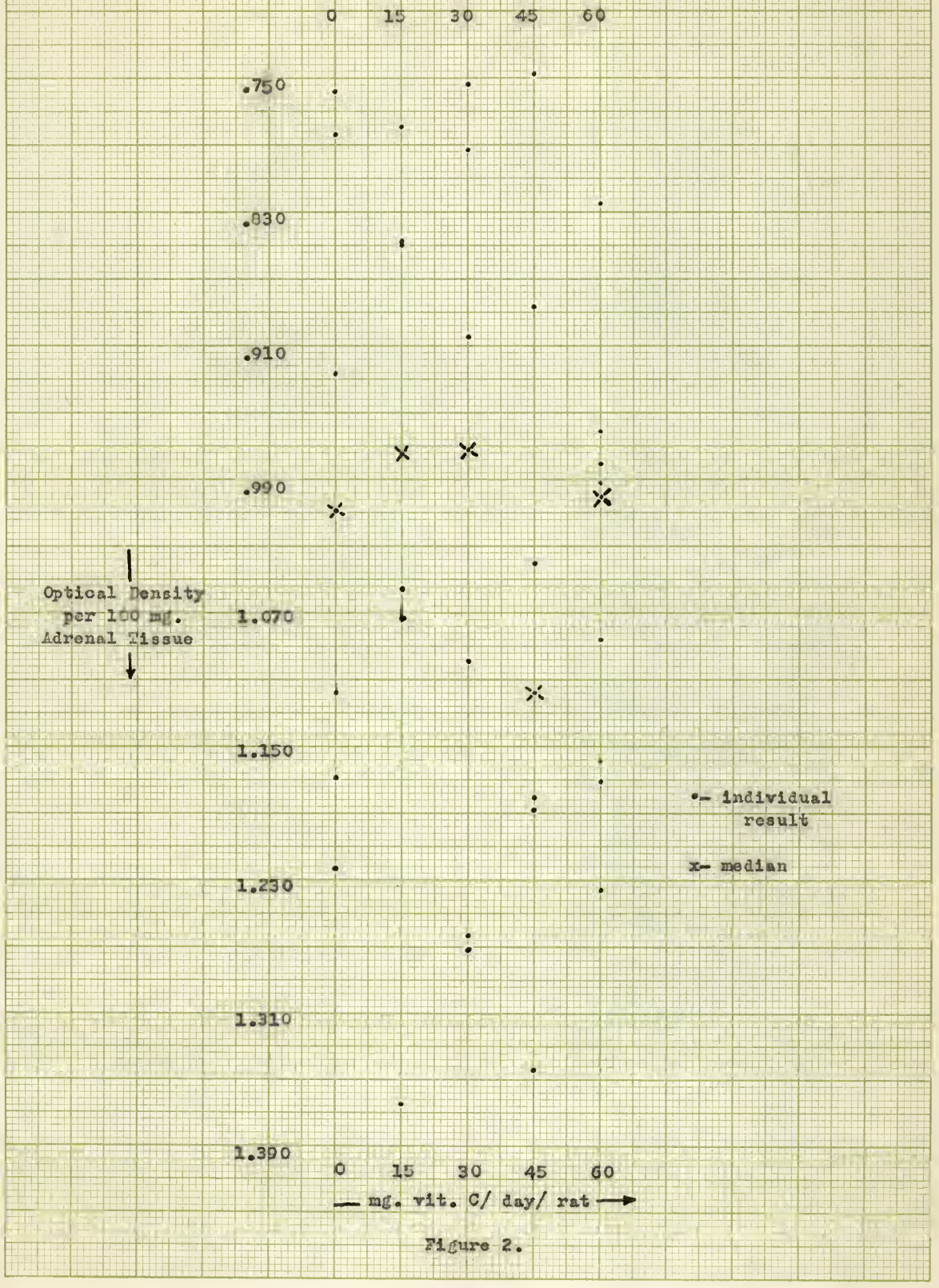

0
15
30
45
60
.750
.830
.910
.990
1.070
1.150
1.230
1.310
1.390
Optical Density per 100 mg. Adrenal Tissue
•- individual result
x- median
0
15
30
45
60
— mg. vit. C/ day/ rat ⟶

Figure 2.

According to Dr. H. C. Fryer, Kansas State College statistician, it can be said that the variation between individual results on the same levels do not permit quantitative estimation of the differences in reduction for the levels. However, the results do warrant the conclusion of a definite trend in the aqueous fraction that is, as dietary vitamin C increased, reducing power of the extract decreased. If the individual results furthest from the medium on each level had not occurred, the trend would not be altered.

EXPERIMENTAL RESULTS - PHASE II

The individual results are too widely spaced to permit quantitative estimation of the differences in recovery time at the 5, 10, and 15 mg. /day/rat levels. However, 12 of 18 rats receiving vitamin C supplement benefitted in recovery time when compared to control animals. Those receiving no vitamin C required an average of 79 seconds more recovery time under the second set of conditions. Those receiving the supplement of the vitamin required only 42 seconds more (Tables 3 and 4).

DISCUSSION
PHASE I

There is a question of the identity of the substance (s) in the aqueous fraction which causes the reduction of phosphomolybdic acid. It appears that increased vitamin C intake results in lowered levels of this substances (s).

The substance(s) is ether-chloroform soluble but more soluble in dilute aqueous alkali. The solubility of the adrenal hormones in these solvents is very small, if at all. However, desoxycorticosterone is believed to occur in the glycoside form. These glycosides are freely soluble in water and are somewhat soluble in ether-chloroform solution.

It is probable that traces of the steroids are soluble in the water. However, most of them are more soluble in the lipid fraction than in the aqueous portion. Cortisone is a possible exception. It is slightly more soluble in water. It may be noted that some of these steroids are present in urine, and are in some form, at least, water soluble.

DISCUSSION
PHASE II

Differences in response between individual animals do not permit distinguishing between the effects on the three supplemented levels of the vitamin. However, two-thirds of the animals with supplementary vitamin C intake benefitted in recovery time following etherization compared to control animals. These results are in accord with work showing

vitamin C is beneficial to animals under various stresses (1, 2) and with other findings indicating some connection between ascorbic acid and the biosynthesis of cortical hormone (10).

SUMMARY - PHASE I

The reduction of phosphomolybdic acid to molybdenum blue by steroids of the adrenocortical class was used to test the effect of increased dietary ascorbic acid upon the oxy-steroid content of this gland.

Increased ascorbic acid intake resulted in a lowered reducing capacity of the aqueous fraction formed by washing an ether-chloroform layer with dilute alkali. The ether-chloroform extract showed no significant difference at varied levels of the vitamin.

SUMMARY - PHASE II

Rats were fed no dietary supplement of ascorbic acid for 25 days, etherized and their recovery times noted. Afterwards, they were placed on different levels of the vitamin and again etherized. Two-thirds of the rats on vitamin C supplemented diets seem to benefit in recovery as measured by control animals.

CONCLUSIONS

Increased dietary ascorbic acid results in a lowered reducing capacity by the aqueous extract of adrenal tissue, indicating an increase in the oxy-type steroid. This is in accord with a postulated coenzyme role for vitamin C in the production of these steroids.

Increased dietary vitamin C seems to aid rats in recovering from the stress of etherization.

ACKNOWLEDGMENTS

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Finally, I wish to acknowledge the valuable advice concerning our results given by the Kansas State College statistician, Dr. H. C. Fryer, and by Dr. R. Silver, research chemist at Merck and Company, Inc., Rahway, New Jersey.

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APPENDIX

TABLE I
OPTICAL DENSITY READINGS - WATER FRACTION

Mg. Vit. C/day	Adrenal Weight (mg.)	Optical Density	O.D./100 mg. adrenal tissue
0	44.0	.164	.373
0	39.4	.162	.411
0	40.8	.167	.409
0	44.6	.159	.356
0	43.5	.152	.350
0	42.3	.172	.407
0	37.8	.132	.349
0	41.7	.109	.262
			Median - .3645
15	44.6	.120	.269
15	35.2	.136	.386
15	37.2	.142	.382
15	40.3	.114	.283
15	40.3	.133	.330
15	41.1	.163	.396
15	36.1	.122	.338
15	49.0	.152	.310
			Median - .3340
30	45.3	.136	.300
30	38.8	.130	.335
30	41.1	.112	.272
30	40.1	.112	.279
30	43.3	.085	.196
30	41.2	.103	.250
			Median - .2755
45	39.6	.082	.207
45	48.0	.126	.263
45	40.4	.108	.267
45	38.1	.119	.312
45	43.3	.108	.249
45	41.2	.098	.238
			Median - .2560
60	44.7	.089	.199
60	41.1	.090	.219
60	47.2	.128	.271
60	40.7	.099	.243
60	40.0	.101	.252
60	37.3	.090	.241
60	48.0	.142	.330
			Median - .2430

TABLE II
OPTICAL DENSITY READINGS - LIPID FRACTION

Mg. Vit. C/day	Adrenal Weight (mg.)	Optical Density	O.D./100 mg. adrenal tissue
0	42.0	.466	1.109
0	42.3	.392	.927
0	43.5	.508	1.169
0	42.3	.321	.758
0	44.6	.450	1.009
0	39.4	.309	.784
0	41.7	.510	1.223
			Median - 1.009
15	36.1	.352	.975
15	49.0	.382	.779
15	41.1	.441	1.073
15	40.3	.343	.850
15	40.3	.550	1.365
15	39.6	.418	1.056
15	35.2	.299	.849
			Median - .975
30	41.2	.373	.905
30	34.6	.440	1.272
30	40.6	.446	1.099
30	43.3	.343	.792
30	40.1	.389	.972
30	38.8	.490	1.263
30	41.1	.310	.754
			Median - .972
45	44.4	.462	1.041
45	38.1	.450	1.181
45	36.7	.410	1.118
45	46.1	.409	.887
45	39.9	.536	1.344
45	48.0	.570	1.188
45	43.3	.324	.748
			Median - 1.118
60	37.3	.366	.981
60	36.9	.357	.967
60	35.0	.352	1.001
60	42.1	.488	1.159
60	40.0	.330	.825
60	47.2	.468	.992
60	44.7	.485	1.087
60	35.6	.440	1.236
60	41.0	.480	1.171
			Median - 1.001

TABLE III

ETHERIZING RECOVERY TIMES (PHASE II)

Group	Mg. vit. C per day	Recovery time (basal diet) (sec.)	"Correction Factor"	Column 3 ± Column 4 (sec.)	Recovery time (with vit. C) (sec.)	Improvement (sec.)
1	0	255	± 75	330	330	0
1	5	335	± 75	410	339	± 71
1	10	235	± 75	310	250	± 60
1	15	180	± 75	255	235	± 20
2	0	279	±154	433	433	0
2	5	389	±154	543	382	±161
2	10	420	±154	574	510	± 64
2	15	260	±154	414	279	±135
3	0	180	±215	395	395	0
3	5	238	±215	453	284	±169
3	10	285	±215	500	343	±157
3	15	400	±215	615	446	±169
4	0	206	±135	341	341	0
4	5	165	±135	300	342	- 42
4	10	253	±135	388	416	- 28
4	15	202	±135	337	430	- 93
5	0	254	- 45	209	209	0
5	5	232	- 45	187	291	-104
5	10	294	- 45	249	215	± 34
5	15	405	- 45	360	297	± 63
6	0	285	- 15	270	270	0
6	5	340	- 15	325	328	- 3
6	10	405	- 15	390	336	± 54
6	15	315	- 15	300	312	- 12

TABLE IV

(a) Dietary Vitamin C per day prior to second etherizing	(b) Average recovery time - first etherizing (seconds)	(c) Average recovery time - second etherizing (seconds)	(d) Difference (seconds)
0	223	296	73
5	283	322	39
10	315	390	75
15	320	333	13
5, 10 & 15 combined	306	348	42

SOME EFFECTS OF VARYING DIETARY VITAMIN C LEVELS ON THE REDUCING CAPACITY OF THE ADRENAL GLANDS AND THE FUNCTION OF THESE BODIES UNDER STRESS

by

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A number of studies have indicated that vitamin C, ascorbic acid, may play an important role in the biosynthesis of the oxy-type cortical steroids. Further, since these steroids are intimately involved in the general adaptation of an animal to stress, it might be expected that increased dietary ascorbic acid would result in increased ability to survive stress. To test the validity of these hypotheses, we performed two phases of experimentation:

PHASE I - The effect of dietary vitamin C on the level of the oxy-steroids in the adrenal tissue of the albino rat was tested by the reduction of phosphomolybdic acid to molybdenum blue by these steroids. The intensity of the color produced was measured with a colorimeter.

A chloroform extract was made of freshly ground adrenal tissue. This extract was shaken with 0.1 N NaOH (aqueous) and both layers which formed on standing were analyzed by the above method. Although the lipid fraction showed no significant differences at the various dietary levels of the vitamin, the alkaline aqueous solution indicated that as dietary ascorbic acid increased, cortical steroidal reducing power in this fraction, at least, decreased.

PHASE II - Rats were maintained for 25 days with no supplementary vitamin C. They were then etherized and their recovery times noted. After this, the animals were placed on various dietary levels, namely, 0, 5, 10 and 15 mg./day. Again they were etherized and their recovery times noted. Those receiving no vitamin C required an average of 79 seconds more recovery time under the second set of conditions. Those receiving the supplement of the vitamin required only 42 seconds more. Variation was too wide to cite any difference in improvement among the top three levels of vitaminization.